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Alcohol Dehydrogenase from *Methylobacterium organophilum*

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The alcohol dehydrogenase from *Methylobacterium organophilum*, a facultative methane-oxidizing bacterium, has been purified to homogeneity as indicated by sodium dodecyl sulfate-gel electrophoresis. It has several properties in common with the alcohol dehydrogenases from other methylotrophic bacteria. The active enzyme is a dimeric protein, both subunits having molecular weights of about 62,000. The enzyme exhibits broad substrate specificity for primary alcohols and catalyzes the two-step oxidation of methanol to formate. The apparent Michaelis constants of the enzyme are 2.9×10^{-5} M for methanol and 8.2×10^{-5} M for formaldehyde. Activity of the purified enzyme is dependent on phenazine methosulfate. Certain characteristics of this enzyme distinguish it from the other alcohol dehydrogenases of other methylotrophic bacteria. Ammonia is not required for, but stimulates the activity of newly purified enzyme. An absolute dependence on ammonia develops after storage of the purified enzyme. Activity is not inhibited by phosphate. The fluorescence spectrum of the enzyme indicates that it and the cofactor associated with it may be chemically different from the alcohol dehydrogenases from other methylotrophic bacteria. The alcohol dehydrogenases of *Hyphomicrobium* WC-65, *Pseudomonas methanica*, *Methylosinus trichosporium*, and several facultative methylotrophs are serologically related to the enzyme purified in this study. The enzymes of *Rhodopseudomonas acidophila* and of organisms of the *Methylococcus* group did not cross-react with the antiserum prepared against the alcohol dehydrogenase of *M. organophilum*.

Bacteria capable of using one-carbon compounds to provide their carbon and energy requirements for growth have been described previously (16, 18, 29). These organisms can be grouped into two categories. Obligate methylotrophs usually utilize methane and are absolutely dependent on one-carbon compounds as growth substrates. Facultative methylotrophs can grow on more complex organic substrates such as succinate or glucose in addition to one-carbon compounds. Most cannot oxidize methane.

An alcohol dehydrogenase (also called methanol dehydrogenase) plays a major role during growth of these organisms on methane or methanol; the enzyme catalyzes the oxidation of methanol to formate. Alcohol dehydrogenases have been purified from several facultative and obligate methylotrophic bacteria (2, 3, 8, 14, 21, 22, 26). All have molecular weights in the range of 120,000 to 140,000. Absorption spectra have revealed maxima at 280 and 350 nm. Enzymatic activity was dependent on the presence of phenazine methosulfate (PMS) as an artificial electron carrier; ammonia was required for activity. Nicotinamide adenine dinucleotide (NAD^+) was not reduced by the enzymes. High pH optima were observed. The enzymes exhibited broad substrate specificity for primary aliphatic alcohols and were also capable of oxidizing formaldehyde to formate. Immunological and biochemical studies comparing the enzymes of an obligate methylotroph and a facultative organism have suggested a close relationship between these organisms in spite of differences in their carbon assimilation pathways (14, 15).

We are currently studying a facultative methylotroph which can grow on methane, methanol, and more complex organic substrates. The alcohol dehydrogenase of this organism has been purified and biochemically characterized. Serological studies using crude extracts of several methylotrophs and antiserum prepared against this enzyme have been employed to provide information on the taxonomic position of this microorganism.

MATERIALS AND METHODS

Organisms and growth conditions. *Methylobacterium organophilum* strain XX (17), ATCC 27886, was used as the source of the alcohol dehydrogenase. Other facultative methane oxidizers were isolated from

Lake Mendota, Madison, Wis., using the enrichment technique of Patt et al. (16). Single clones that grew on plates in the presence of methane were streaked on nutrient agar plates; the resultant colonies were transferred and maintained on methane. Other methylotrophs were kindly provided by the following people: *Methylococcus capsulatus* and *Pseudomonas methanica* by D. W. Ribbons, University of Miami School of Medicine, Miami, Fla.; *Methylococcus* strains 999 and B1 by Shell Research Limited, Sittingbourne, Kent, England; *Methylosinus trichosporium* OB3b by R. N. Patel, Corporate Research Laboratories, Exxon Research and Engineering Co., Linden, N.J., and by R. W. Whittenbury, Warwick University, Coventry, England; *Rhodopseudomonas acidophila* 10050 by J. R. Quayle, University of Sheffield, Sheffield, England; *Hyphomicrobium* WC-65 and the facultative methanol oxidizer JB-1 by E. Bellion, University of Texas, Arlington.

R. acidophila was grown photosynthetically in basal medium (19) with 0.2% methanol (vol/vol) added. All other organisms were grown in liquid NMS medium (16) at 30°C on rotary shakers, except *Hyphomicrobium* WC-65, which was incubated without shaking. All *Methylococcus* strains, *P. methanica, M. trichosporium, M. organophilum*, and some facultative methane oxidizers were incubated under a 75% methane–25% air atmosphere. Methanol was filter sterilized and added to the medium to a final concentration of 0.5% (vol/vol) when growing *Hyphomicrobium* WC-65, organism JB-1, and other facultative methylotrophs. *M. organophilum* was grown in a 14-liter New Brunswick Microferm fermentor at 30°C with continuous aeration on 0.5% methanol (vol/vol).

Cells were harvested by centrifugation in the middle of the exponential phase of growth, washed three times with 50 mM phosphate buffer (pH 7.0), and stored at −20°C.

Crude extracts. Cells were thawed, resuspended in twice their weight of the phosphate buffer, and disrupted by three passes through a French pressure cell at 18,000 lb/in^2. The extracts were centrifuged at 20,000 × *g* for 20 min at 4°C; the supernatant fractions were stored on ice until assayed.

Assays of the alcohol dehydrogenase. Alcohol dehydrogenase was assayed polarigraphically using a Rank oxygen electrode (Rank Bros., Biosham, Great Britain). The normal assay mixture contained (in 1.0 ml): 100 μmol of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 9.0) and 95 μmol of sodium carbonate (pH 9.0), 0.33 μmol of PMS, 20 μmol of NH_4Cl, 12.4 μmol of redistilled methanol or 10 μmol of formaldehyde, and enzyme. When determining the substrate specificity of the enzyme, 8 to 10 μmol of each compound indicated in Table 2 was added to the assay mixture instead of methanol or formaldehyde. Purified enzyme was used for all experiments unless otherwise noted. Assays were run at 40°C unless otherwise noted. The reaction was started by addition of the substrate. A unit of activity is defined as the amount of enzyme required to give a rate of 1 μmol of O_2 consumed per min. Specific activity is expressed as micromoles of O_2 consumed per minute per milligram of protein.

Enzyme activity was also monitored spectrophotometrically by following the reduction of 2,6-dichlorophenolindophenol at 600 nm or the reduction of purified *c*-type cytochrome from *M. organophilum* at 520 nm. The assay mixture and procedure used were those described by Anthony and Zatman (2).

Purification of the alcohol dehydrogenase. The crude extract of *M. organophilum* was adjusted to pH 4.0 by the slow addition of 0.5 N HCl at 4°C with constant stirring. This suspension was centrifuged at 90,000 × *g* for 1 h at 4°C to remove the heavy precipitate. The supernatant fraction was adjusted to pH 7.0 by the addition of 0.5 N NaOH at 4°C.

The neutralized enzyme preparation was dialyzed overnight at 4°C against the volume of ammonium sulfate-saturated 10 mM Tris buffer required to make the final $(NH_4)_2SO_4$ saturation 85%. The precipitate formed was removed by centrifugation at 20,000 × *g* at 4°C for 20 min and was suspended in a minimum volume of 10 mM Tris-hydrochloride buffer (pH 8.0). $(NH_4)_2SO_4$-saturated Tris buffer was added dropwise to the enzyme preparation with constant stirring at 4°C until a final saturation of 55% was reached. After low-speed centrifugation, the supernatant fraction was dialyzed against 1,000 volumes of the Tris-hydrochloride buffer.

One-half of the dialyzed sample (3.5 ml) was applied to a column (1.3 by 25 cm) of diethylaminoethyl-cellulose (Whatman DE52) equilibrated with 10 mM Tris-hydrochloride buffer (pH 8.0); 100 ml of buffer was passed through the column until no further protein was eluted, as indicated by absorbance at 280 nm. A linear KCl gradient (200 ml, 0 to 0.4 M) was applied. The yellow-colored alcohol dehydrogenase eluted at 0.1 M KCl. Fractions with high enzyme activity were pooled and dialyzed against the $(NH_4)_2SO_4$-saturated Tris buffer.

The precipitate was removed by centrifugation, resuspended in a minimum volume of the Tris-hydrochloride buffer, and applied to a Bio-Gel A 1.5-m, 100- to 200-mesh column (1.3 by 35 cm). Tris-hydrochloride buffer was passed through the column. Fractions with the highest specific activity were pooled and stored under N_2 at −20°C.

During purification, the enzyme preparation was protected from strong light and was constantly kept in the presence of N_2.

The requirement of the enzyme for ammonia was examined under enzyme preparations purified in the absence of ammonia. The acid precipitation step was followed by diethylaminoethyl-cellulose chromatography. Fractions with the highest specific activity were assayed immediately and stored without further concentration. All solutions and buffers were prepared with distilled water that had been further deionized using a strong anion-strong cation mixed-resin bed provided by the Continental Deionized Water Service, Madison, Wis.

Cytochrome purification. The soluble *c*-type cytochrome was purified using method I described by Anthony (1) with one modification. Cytochrome fractions were concentrated by dialysis against 25% polyethylene glycol 20,000 instead of by ultrafiltration.

Protein homogeneity. The homogeneity of the enzyme and cytochrome preparations was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using the method of Laemmli (12). Gels were stained using a series of isopropyl alcohol-

acetic acid-water-Coomassie brilliant blue solutions as described by Fairbanks et al. (7) and were destained in 10% acetic acid (vol/vol).

Spectrophotometry. Absorption spectra were determined using a Cary 15 spectrophotometer. Fluorescence spectra of the active enzyme were measured in distilled water using an Aminco-Bowman spectrophotofluorometer.

Molecular weight determinations. The molecular weight of the active enzyme was determined by the slope-molecular weight method of Hedrick and Smith (9). The enzyme and protein standards were subjected to electrophoresis in 6, 7, 8, and 9% polyacrylamide gels. The slope of the plot of logarithm of relative mobility versus percent gel was plotted against the molecular weight of the proteins. Hemoglobin (68,000) (11), enolase (88,000) (20), lactic dehydrogenase (135,000) (9), and catalase (250,000) (9) were used as standards. The subunit molecular weight was determined by SDS-gel electrophoresis, as described by Weber and Osborn (27). Standards included cellulase (76,000) (30), bovine serum albumin (68,000) (27), glyceraldehyde phosphate dehydrogenase (36,000) (27), and trypsin (23,300) (27).

The cytochrome was also stained for heme iron using the procedure of Clarke (5). The active alcohol dehydrogenase was located in polyacrylamide gels using the nitroblue tetrazolium coupled assay described by Wadzinski and Ribbons (26).

Immunological methods. Antiserum to the purified alcohol dehydrogenase was prepared using random-bred New Zealand rabbits. Two injections of 1 mg each were given intramuscularly at 1-week intervals. Two weeks later, three intravenous injections of 0.2 mg of antigen each were given at 2-day intervals. The rabbits were bled by marginal ear puncture 1 week later and for the following weeks. When necessary, booster doses of 1 mg of enzyme in Freund adjuvant were given intramuscularly.

Cross-reactivity was determined by the Ouchterlony double-diffusion technique (23). Equal amounts of methanol-oxidizing activity were added to each antigen-containing well. Reactions were carried out at 20°C and interpreted after 24 h of incubation.

Analytical procedures. Formate was determined by the method of Wadzinski and Ribbons (26), using the NAD-linked formate dehydrogenase present in crude extracts of *M. organophilum*. The assay volume was changed to 3.0 ml. Appropriate formate standards were included.

Protein was determined by the Folin phenol reagent method (13). Crystalline bovine serum albumin (Sigma, fraction V) was used as the standard; absorbance was measured at 660 nm on Gilford 2000 spectrophotometer.

Materials. Formaldehyde was prepared by autoclaving an aqueous solution containing a weighed amount of paraformaldehyde under an N_2 atmosphere in a sealed vial.

Tris-hydrochloride, 2,6-dichlorophenolindophenol, PMS, nitroblue tetrazolium, and the standards for electrophoresis were obtained from the Sigma Chemical Co., St. Louis, Mo. Whatman DE52 was obtained through H. Reeve Angel and Co. Ltd., Clifton, N.J. Bio-Gel A, acrylamide, and methylene bisacrylamide were obtained from Bio-Rad Laboratories, Richmond, Va.

RESULTS

Purification of the cytochrome. A soluble *c*-type cytochrome was obtained that appeared identifical to those previously described by Anthony (1) and Tonge et al. (24). The absorption spectrum had characteristic α, β, and γ peaks at 549 to 552 nm, 519 to 521 nm, and 415 to 417 nm, respectively. The (reduced plus CO) minus reduced difference spectrum exhibited an absorption peak at 535 nm and a corresponding trough at 549 nm. SDS-polyacrylamide gel electrophoresis of the final product indicated the presence of a single heme iron protein with a molecular weight of 21,500.

Purification of the alcohol dehydrogenase. Table 1 summarizes the purification of the alcohol dehydrogenase from *M. organophilum*. A 20-fold purification and a 25% yield were achieved. A single band was obtained when the resultant protein was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1). The purification procedure represented a modification of that used by Patel et al. (14) to purify alcohol dehydrogenases from methylotrophic bacteria. Elimination of an ultracentrifugation step and utilization of a different ammonium sulfate fractionation procedure resulted in both a higher final specific activity and a greater yield. The equilibration of the diethylaminoethyl column with a lower concentration buffer, 10 mM rather

TABLE 1. *Purification of the alcohol dehydrogenase from M. organophilum*

Step	Vol (ml)	Total U[a]	Protein (mg/ml)	Sp act[b]	% Yield	Fold purification
Crude extract	46.1	136	42.0	0.07	100	
Reneutralized enzyme fraction	40.5	67	8.7	0.19	49.3	2.7
$(NH_4)_2SO_4$ fraction (55–85% saturation)	7.0	48	12.3	0.56	35.3	8.0
Diethylaminoethyl-cellulose column	27.3	44	1.7	0.94	32.4	13.4
Bio-Gel column	19.9	34	1.2	1.43	25.0	20.4

[a] Expressed as micromoles of O_2 consumed per minute.

[b] Expressed as micromoles of O_2 consumed per minute per milligram of protein.

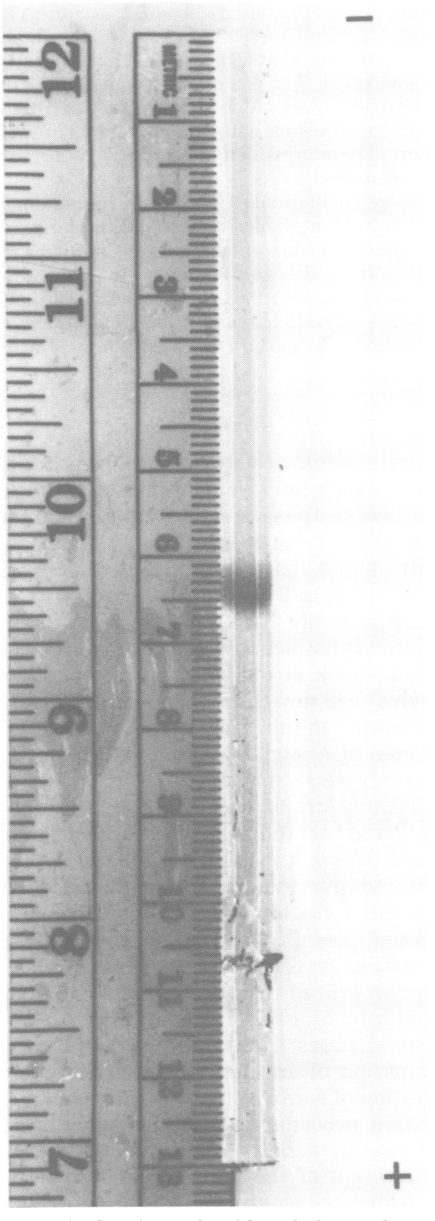

FIG. 1. *SDS-polyacrylamide gel electrophoresis of the purified alcohol dehydrogenase. Electrophoresis was performed on 9% gels. Migration occurred from top to bottom.*

than 20 mM Tris-hydrochloride, permitted retention of the enzyme on the column while the major contaminant, the cytochrome, eluted off.

Enzymatic activity. The alcohol dehydrogenase could be assayed polarigraphically or spectrophotometrically coupled to the reduction of dichlorophenolindophenol or the purified cytochrome; however, the polarigraph assay was more sensitive and reproducible. PMS was required for activity of the purified enzyme. Activity was not PMS dependent until after ammonium sulfate fractionation of the extract. Addition of PMS during assay of the crude extract resulted in a twofold increase in activity. NAD^+ had no effect on enzyme activity.

(i) Substrates. All primary aliphatic alcohols tested were oxidized by the enzyme; the shorter alcohols were oxidized at rates comparable to that of methanol oxidation (Table 2). Secondary, tertiary, and cyclic alcohols also served as substrates. Formaldehyde was oxidized at half the rate of methanol.

(ii) Stoichiometry. The data in Table 3 suggest the complete conversion of methanol or formaldehyde to formate. The molar yields of reduced NAD formed in the formate dehydrogenase assays (and therefore the amounts of formate consumed in the assays) were equal to the original quantities of methanol and formaldehyde provided as substrates for the alcohol dehydrogenase. The amounts of oxygen consumed during the oxidations of methanol and

TABLE 2. *Substrate specificity of the alcohol dehydrogenase*

Substrates	Oxidation rate (%) compared to methanol oxidation
Ethanol, 1-propanol, 1-pentanol, 1-octanol	90–110
Formaldehyde, ethanolamine	40–50
Isobutanol, *t*-butanol, cyclohexanol	5–10

TABLE 3. *Stoichiometry of methanol and formaldehyde oxidation by the alcohol dehydrogenase from M. organophilum*

Substrate	Amt (nmol) added to alcohol dehydrogenase assay[a]	Total nmol of O_2 consumed	nmol of NAD reduced (formate present)[b]
Methanol	246	239	ND[c]
Methanol	625	543	533
Formaldehyde	250	116	ND
Formaldehyde	1,000	ND	852
Formate[d]	500	0	423

[a] Substrate was incubated with 50 U of enzyme for 30 min or until no further O_2 consumption occurred.

[b] NAD was reduced when crude extract containing an NAD-linked formate dehydrogenase was added, if formate was present.

[c] ND, Not determined.

[d] Included as a control to determine recovery of formate during this experiment.

formaldehyde that were allowed to go to completion indicated that formate was the only product of the reaction.

(iii) pH optimum. The pH optimum for enzyme activity was 10.0 to 10.5. The assay was not accurate at higher pH values since oxygen consumption (or dye reduction) occurred in the absence of enzyme at pH values higher than 10.5. Figure 2 shows the progress of reactions run at several pH's. At all pH's tested, the reaction rate was neither linear nor maximum immediately after methanol addition. The length of time that was required for it to reach a linear, maximum rate decreased with increasing pH. Assays run at pH values of 7.5 and 8.0 showed no oxygen consumption for at least 5 min and did not reach maximum activity until after 11 min.

(iv) Effectors. Ammonia was not required for, but did stimulate the activity of freshly prepared enzyme. The optimum concentration for stimulation was 20 mM NH_4Cl. A good enzyme preparation showed a 2.5- to 4.5-fold stimulation. The amount of stimulation increased upon storage of the enzyme due to a gradual loss of the ammonia-independent activity. Methylamine enhanced activity to a lesser degree. NaCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, or $LiCl_2$ did not affect activity. The addition of KCl to an assay mixture containing NH_4Cl resulted in partial reversal of the observed stimulation (Fig. 3).

Cyanide was found to inhibit the enzyme; 0.5 mM NaCN caused a 25% decrease in methanol-oxidizing activity; 200 mM phosphate did not inhibit activity. ATP, ADP, AMP, phosphoenolpyruvate, methane, glyoxylate, or glycolate did not affect the enzyme.

(v) Kinetics. The apparent Michaelis-Menten constants for methanol and formaldehyde were determined using the double-reciprocal plot method of Lineweaver and Burk. A K_m of 2.9×10^{-5} M was found for methanol; the presence of NH_4Cl did not affect this value (Fig. 4). The V_{max} for methanol oxidation increased 2.5-fold in its presence. A K_m of 8.2×10^{-5} M was found for formaldehyde.

(vi) Stability of the purified enzyme. Purification of the enzyme in the presence of strong light and air resulted in a preparation with low activity that was unstable to storage. The enzyme was also unstable when stored in a dilute solution (<5 mg/ml). When prepared under N_2 and protected from strong light, the enzyme retained up to 70% of its ammonia-stimulated activity for up to 4 months if stored at −20°C. The ammonia-independent activity was lost after storage at −20°C for 2 weeks.

Physical characteristics. (i) Molecular weight. The active enzyme had a molecular weight of 135,000 ± 5,000 as determined by its

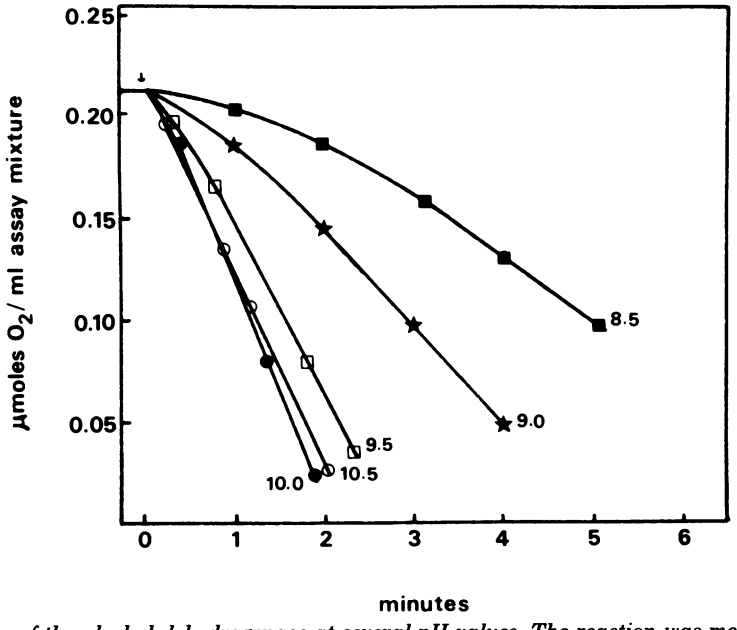

FIG. 2. *Assays of the alcohol dehydrogenase at several pH values. The reaction was measured as micromoles of O_2 consumed per milliliter of assay mixture per minute. The reaction was started by the addition of methanol at the time indicated by the arrow.*

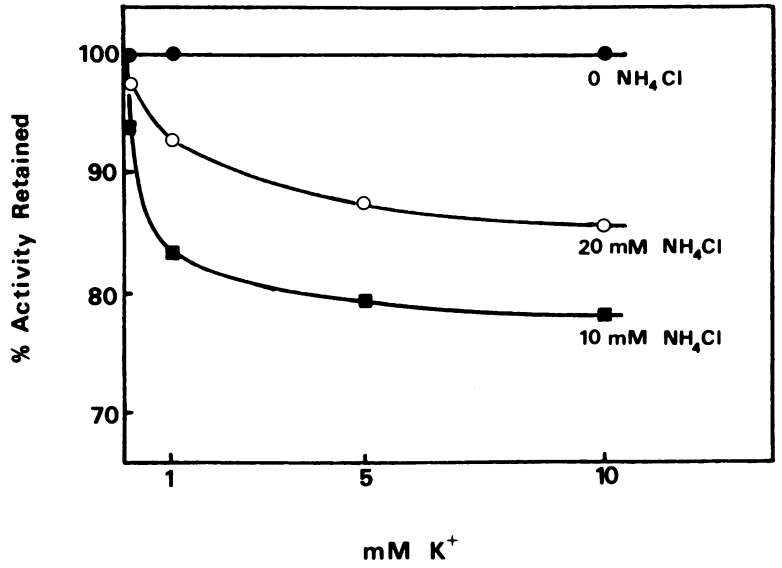

FIG. 3. *Effect of KCl on the alcohol dehydrogenase in the presence of varying concentrations of ammonia. Symbols:* (●) *no NH_4Cl added, 100% activity equaled 1.7 nmol of O_2 consumed per min;* (■) *10 mM NH_4Cl added, 100% activity equaled 6.9 nmol of O_2 consumed per min;* (○) *20 mM NH_4Cl added, 100% activity equaled 8.6 nmol of O_2 consumed per min.*

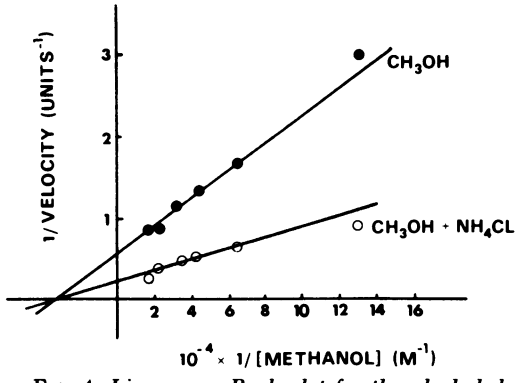

FIG. 4. *Lineweaver-Burk plot for the alcohol dehydrogenase from M. organophilum. Methanol oxidation in the presence and absence of 20 mM NH_4Cl was measured the same day that the enzyme was purified.*

relative mobility in polyacrylamide gels of increasing acrylamide percentages. A single band was obtained after electrophoresis of the enzyme on SDS-polyacrylamide gels, corresponding to a molecular weight of 62,000. This suggests the presence of two subunits of the same molecular weight in the active enzyme.

(ii) Absorption spectrum. The purified enzyme exhibited absorption maxima at 280 and 345 nm with little absorption above 420 nm.

(iii) Fluorescence spectrum. The purified enzyme exhibited an excitation maximum at 285 to 290 nm and an emission maximum at 355 to 360 nm. Fluorescence was maximum over a pH range of 2 to 7; 0.1 N NaOH quenched the emission 90%.

Serological studies. A single precipitin line was formed in Ouchterlony plates when the crude extract of *M. organophilum* was cross-reacted with the antiserum prepared against the purified alcohol dehydrogenase of the same organism (Fig. 5A). This indicated specificity for the alcohol dehydrogenase. Figure 5 shows representative reactions obtained during the serological studies using crude extracts of several methylotrophs; Table 4 summarizes these results. The extracts of *R. acidophila, Methylococcus capsulatus, Methylococcus* strains 999 and B1, and one facultative methane oxidizer did not cross-react with the antiserum even though methanol-oxidizing activity was present. The extracts of facultative methylotrophs, including *M. organophilum* and organism JB-1, and *P. methanica* formed precipitin bands that fused with each other to give reactions of complete identity. The extract of *M. trichosporium* formed a precipitin band that fused with those of the purified enzyme and the extracts of the facultative methane oxidizers, but spurs were formed with the precipitin bands of *P. methanica* and organism JB-1. The extract of *Hyphomicrobium* WC-65 formed spurs with all other cross-reacting extracts.

Presence of the enzyme in methane-

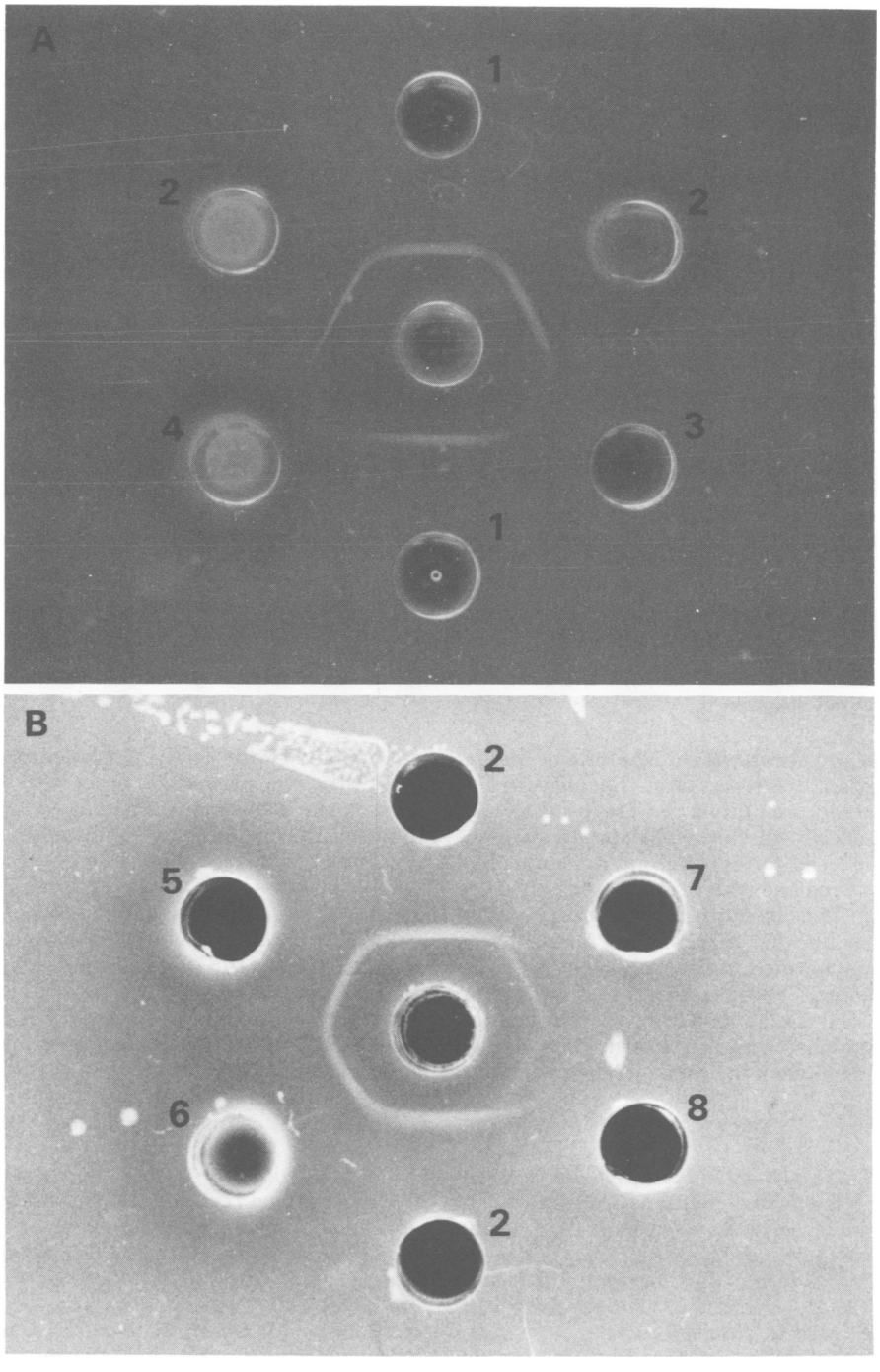

FIG. 5. *Immunodiffusion studies showing representative cross-reactions between the extracts of several methylotrophs. Equal units of methanol-oxidizing activity of the extracts were placed in the outer wells of the Ouchterlony plates; antiserum prepared against the purified alcohol dehydrogenase from M. organophilum was placed in the center well. (A) Cross-reactions of M. organophilum: (1) purified enzyme; (2) extract of methanol-grown cells; (3) extract of methane-grown cells; (4) an extract of Escherichia coli run as a control. (B) Cross-reactions of extracts of several methylotrophs illustrating the different patterns of reaction: (2) methanol-grown M. organophilum; (5) organism JB-1; (6) P. methanica; (7) M. trichosporium; (8) Hyphomicrobium WC-65.*

TABLE 4. *Serological studies of methylotrophic bacteria*

Organism	Growth substrate	Sp act[a]	Cross-reactivity[b]	Identity pattern[c]					
				M.o.	fs.5	JB-1	*P.m.*	OB3b	WC-65
R. acidophila 10050	Light + CH_4	0.005	None						
M. capsulatus	CH_4	0.039	None						
Methylococcus 999	CH_4	0.015	None						
Methylococcus B1	CH_4	0.003	None						
Organism fs.11	CH_4	0.009	None						
M. organophilum XX	CH_3OH	0.049	+	i	i	i	i	i	p
	CH_4	0.349	+	i	i	i	i	ND	ND
P. methanica	CH_4	0.140	+	i	i	i	i	p	p
Organism JB-1	CH_3OH	0.068	+	i	i	i	i	p	p
Organism fs.4P	CH_3OH	0.058	+	i	i	i	i	ND	ND
Organism fs.104	CH_3OH	0.060	+	i	i	i	i	ND	ND
Organism fs.7	CH_4	0.472	+	i	i	ND	i	i	i
M. trichosporium OB3b	CH_4	0.034	+	i	i	p	p	i	p
Hyphomicrobium WC-65	CH_3OH	0.026	+	p	p	p	p	p	i

[a] Specific activity of crude extracts is expressed as micromoles of O_2 consumed per minute per milligram of protein at 30°C with methanol as the substrate.

[b] Equal amounts of enzyme activity were added to each well containing antigen.

[c] The precipitin reaction of the organism was compared to the reactions of extracts of other organisms that are representative of different groups of methylotrophs. *M.o.*, *M. organophilum*, a facultative methane oxidizer; fs.5, organism fs.5, a facultative methane oxidizer; JB-1, organism JB-1, a facultative methanol oxidizer; *P.m.*, *P. methanica*, an obligate, type I methane oxidizer; OB3b, *M. trichosporium* OB3b, an obligate type II methane oxidizer; WC-65, *Hyphomicrobium* WC-65, a methanol oxidizer that can use nitrate as the terminal electron acceptor under anaerobic conditions. i, Identity (fusion of precipitin arcs); p, partial identity (formation of spurs); ND, not determined.

grown *M. organophilum*. The rate of methanol oxidation by extracts of methane-grown cultures of *M. organophilum* was greater than that of methanol-grown cultures. Extracts of methane-grown cells formed a faint precipitin line when cross-reacted with the prepared antiserum (Fig. 5A). This line fused with the precipitin lines of the purified enzyme and of the extracts of methanol-grown cells. Polyacrylamide gel electrophoresis of extracts of cells grown on methane and methanol and of the purified enzyme revealed the same pattern when stained for PMS-dependent methanol-oxidizing activity.

DISCUSSION

The NAD-independent alcohol dehydrogenase from a facultative methane-oxidizing bacterium is similar in size and subunit composition to the alcohol dehydrogenases from other methylotrophic bacteria. This enzyme, molecular weight 135,000, consists of two subunits with molecular weights of 62,000 each.

The alcohol dehydrogenase from *M. organophilum*, like the alcohol dehydrogenases from other methylotrophs, exhibits broad substrate specificity. It oxidizes short-chain primary alcohols nearly as quickly as methanol. In addition, it can slowly oxidize branched and cyclic alcohols, some of which have not been reported as substrates for previously described enzymes. The enzyme's Michaelis constants, 2.9×10^{-5} M for methanol and 8.2×10^{-5} M for formaldehyde, are similar to those reported for other methylotrophic systems, with the exceptions of *M. capsulatus* Texas strain (14) and *R. acidophila* (21).

PMS is required for activity of the purified enzyme. Unlike the other alcohol dehydrogenases, this enzyme does not have an absolute requirement for ammonia or methylamine for enzyme activity. The stimulation of activity by ammonia and the development of ammonia dependence after enzyme storage suggests the ammonia may cause a conformational change in the enzyme.

Only two inhibitors of methanol-oxidizing activity have been reported. Phosphate inhibition of methanol oxidation in *M. trichosporium* and in *P. methanica* permitted investigators to measure methane oxidation by the accumulation of methanol (10, 24). Phosphate also inhibited methanol oxidation in the facultative methanol oxidizer *Pseudomonas* AM1 (1). Since methanol oxidation in *M. organophilum* was not inhibited by up to 200 mM phosphate in assays of the crude extract or the purified enzyme, this enzyme appears to be different from these other enzymes. The enzyme was inhibited by cyanide,

a property shared with the methanol-oxidizing system of *M. capsulatus* (6).

The NAD-independent alcohol dehydrogenases exhibit high pH optima, usually around pH 9.0. The optimum for the enzyme purified in this study was higher. The enzyme of *Pseudomonas* C has also been reported to have a higher optimum, pH 10.4 (8).

The absorption spectrum of the purified enzyme revealed the same maxima at 280 and 350 nm that have been reported for other alcohol dehydrogenases. The active enzyme was fluorescent, a property that was not observed for the enzyme from *Pseudomonas* M27 (4) and not previously reported for the enzymes from other methylotrophs. Anthony and Zatman (4) and Sperl and his co-workers (G. T. Sperl, H. S. Forrest, and D. T. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P61, p. 151) have reported the presence of a fluorescent cofactor associated with the alcohol dehydrogenases from several methylotrophic bacteria. The cofactor exhibited an excitation-emission pair at 350 and 450 nm. The fluorescence spectrum of the active enzyme from *M. organophilum* does not coincide with the spectrum of this cofactor, suggesting that a different molecule may be responsible for fluorescence in the enzyme from *M. organophilum*.

M. organophilum is a gram-negative, polarly flagellated, aerobic rod that fits classification as a type II methylotroph. Because of its ability to grow on methane in addition to methanol, dicarboxylic acids, and monosaccharides, one might predict a taxonomic position between the obligate and facultative methylotrophs. The serological studies coincide with this prediction.

R. acidophila is a photosynthetic bacterium. It is not a true methylotrophic organism, since methanol is not used as its energy source. Its alcohol dehydrogenase has a low affinity for methanol (21). Its lack of cross-reactivity is consistent with the difference in this enzyme's function and biochemical properties. Organisms of the *Methylococcus* type are obligate, coccoid methylotrophs. They have recently been recharacterized as type X rather than type I organisms because of the presence of ribulose diphosphate carboxylase in *M. capsulatus* (R. Whittenbury, personal communication). Wadzinski and Ribbons reported that the methanol-oxidizing enzyme in *M. capsulatus* was membrane bound (26). Antiserum prepared against the purified enzyme from *M. capsulatus* was very specific and did not cross-react with the enzymes from facultative methylotrophs (15). The current study also suggests that serologically different alcohol dehydrogenases exist in the *Methylococcus* organisms and the facultative methylotrophs. One yellow facultative methane oxidizer, organism fs.11, also possesses a serologically different enzyme. This organism does not grow on methanol.

The "pseudomonad" type of methylotrophic bacteria, whether obligate or facultative, have serologically similar alcohol dehydrogenases. Organism 4P, a type I facultative methylotroph (A. M. Pilch, unpublished data), and *P. methanica*, a type I obligate methylotroph, are included in this group even though their carbon assimilation pathways and guanine-plus-cytosine contents differ from the rod-shaped methylotrophs. The facultative methane oxidizers, including *M. organophilum*, and the facultative methanol oxidizer, organism JB-1, are pink-pigmented, rod-shaped organisms having guanine-plus-cytosine contents in the range of 70 to 75% (A. M. Pilch, unpublished data).

M. trichosporium is an obligate, type II organism. Its enzyme possesses some antigenic determinants in common with the enzyme of the pseudomonad type of methylotroph. The fusion of the precipitin lines from *M. trichosporium* and the facultative methane oxidizers, but not from *P. methanica* or organism JB-1, suggests a divergence in this group. The serological difference in *P. methanica* coincides with a difference in the resting stages formed by these organisms. *P. methanica* forms an azotobacter-type cyst, *M. trichosporium* an exospore (28). Organism JB-1 differs from other members of this group in that it cannot oxidize methane. *Hyphomicrobium* WC-65, which is morphologically and physiologically distinct since it can use nitrate as a terminal electron acceptor for methanol oxidation, has only a partial complement in its alcohol dehydrogenase of the antigenic determinants that are found in the cross-reacting enzymes of the other methylotrophs.

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